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Polychlorinated diphenyl sulfides can induce ROS and genotoxicity via the AhR-CYP1A1 pathway

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HIGHLIGHTS

- Some types of PCDPSs can activated AhR pathway and then cause overproduction of ROS.
- High dose of PCDPSs can markedly increase expression of *OGG1*, *XRS2*, which indicates that the major form of genotoxicity is oxidation of DNA bases and breaks of DNA strands.
- PCDPSs can increase expression of OGG1, XRS2, indicating that DNA bases oxidation and DNA strands breaks are the major forms.

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ABSTRACT

Polychlorinated diphenyl sulfides (PCDPSs) are a group of chemicals that can interact and activate the aryl hydrocarbon receptor (AhR). Previous studies have shown that PCDPSs can cause oxidative stress in livers. However, information on genotoxicity of PCDPSs is limited. In this study, it is hypothesized that PCDPSs can produce reactive oxygen species (ROS) by activating the AhR and inducing expression of CYP1A1, which subsequently causes genotoxicity. HepG2 cells (transfected with *AhR* and *CYP1A1* siRNA or not) were exposed to six PCDPSs. ROS and expression of five genes were measured to confirm relationships between genotoxicity and signaling along AhR pathway. After 24 h, a significant concentration-dependent ROS was observed. Production of ROS varied with the number of Cl atoms. And the formation of ROS decreases with the increase of the number of Cl atoms, which was consistent with results observed for polychlorinated biphenyls (PCBs). Most of the tested PCDPSs up-regulated expression of CYP1A1 enzyme via signaling AhR pathways. The exposure to 2,3′,4,5-tetra-CDPS at 10 μM up-regulated the CYP1A1 mRNA in HepG2 cells to 29-fold. Expression of *CYP1A1* mRNA was related to the number of substituted Cl, probably due to the stronger ability of more chlorinated PCDPSs to bind to and activate the AhR. However, there was no significant quantitative relationship between expression of CYP1A1 and concentrations of ROS, probably due to other oxidases' influence. Furthermore, PCDPSs also caused induction of *OGG1* and *XRS2* more than 2 times, indicates oxidation of bases and breaks of strands. The transfection of cells with siRNA to silence expression of the *CYP1A1* gene results in ROS at background levels, further supporting the proposed mechanism PCDPSs inducing ROS and DNA damage via the AhR-mediated pathway.

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1. Introduction

Polychlorinated diphenyl sulfides (PCDPSs) comprise a group of chemicals that are structurally analogous to polychlorinated dibenzo(*p*)dioxins. Recently, PCDPSs were reported as priority pollutants due to their persistence and environmental mobility (Mostrag et al., 2010). PCDPSs are lipophilic (Shi et al., 2012) and can therefore accumulate in aquatic organisms and undergo trophic magnification through the food chain. PCDPSs have widespread industrial applications, such as for high-temperature resistant lubricants in gas turbines and steam machines or additives to provide fire-prevention or insulating media, through which they could be released into the environment. PCDPSs occur in dust from metal

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recycling plants (Sinkkonen et al., 1994), gas and fly ash from waste incineration as well as pulp and paper mill wastewater (Sinkkonen et al., 1994). Sediment of the Elbe River contained certain PCDPS congeners (Schwarzbauer et al., 2000). Recently, several congeners of PCDPSs were detected at concentrations from 0.1 to 6.9 ng/g dry mass (dm) in surface sediment and 0.18–2.03 ng/L in surface water along the Yangtze River (Zhang et al., 2014b).

Some studies of toxic potencies of PCDPSs are available. Results of *in vitro* studies showed that several congeners of PCDPSs have antimicrobial and pesticidal activities (Ambrus et al., 2005). Due to the structural similarities between PCDPSs and polychlorinated biphenyls (PCBs), several studies have explored whether the molecular initiating by which PCDPSs could cause adverse effects is via the aryl hydrocarbon receptor (AhR) signaling pathway. These studies found that several congeners of PCDPSs could activate AhR in engineered luciferase reporter gene (LRG) assays based on avian species (Zhang et al., 2014a) such that they had dioxin-like potencies analogous to those caused by PCBs, polychlorinated dibenzo-p-dioxins (PCDDs) or polychlorinated dibenzo furans (PCDFs). A recent transcriptomics study showed that PCDPSs could activate AhR and significantly up-regulate expression of the mixed function mono-oxygenase enzyme, cytochrome P450 1A1 (CYP1A1), which is considered to be a functional bio-marker of activation of the AhR-mediated signaling pathway in mammal cells (Zhang et al., 2016). Furthermore, potencies for up-regulation of expression of CYP1A1 mRNA could be classified and predicted by numbers and locations of substituted Cl atoms, which affected affinity of binding to the AhR. PCDPSs can induce oxidative stress in livers of freshwater fishes and mice, and change the activity of antioxidase (Li et al., 2012; Zhang et al., 2012). Despite the information available, there were still gaps in knowledge of the mechanism by which PCDPSs caused genotoxicity and the molecular mechanism by which oxidative stress is induced by PCDPSs.

Dioxin-like compounds, including the typical congener, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) can activate AhR as a molecular-initiating event (Zhang et al., 2014a). After the initial binding of the ligand to the ligand binding domain of the AhR, compounds dynamically transfer from the cytoplasm to the nucleus and regulate expression of downstream genes, such as *CYP1A1*. Over-expression of CYP1A1 is associated with production of reactive oxygen species (ROS), including superoxide anions and hydrogen peroxide (H_2O_2) (Zangar et al., 2004). Results of a previous study demonstrated that TCDD could increase the level of ROS by up-regulation of CYP1 enzymes via the AhR pathway (Kopf and Walker, 2010). TCDD can induce damage to DNA by ROS production (Kalaiselvan et al., 2016). This finding inspired us to examine whether the PCDPS-activated AhR pathway can be related to overproduction of ROS.

The primary goal of this study was to test the hypothesis that PCDPSs could induce oxidative stress due to their dioxin-like structure and determine whether PCDPSs can cause genotoxicity. Two research steps were conducted to test this hypothesis: First, cells were exposed *in vitro* to PCDPSs and concentrations of ROS were quantified; Next, expression of genes activated along the AhR pathway were determined as well as expression of genes involved in repair of damage to DNA. Finally, siRNA targeting to *AhR* and *CYP1A1* was used to knock down expression of *AhR* or *CYP1A1* to confirm relationships between the AhR pathway and DNA damage.

2. Materials and methods

2.1. Chemicals and solutions

Six congeners of PCDPSs, including 2,3′,4,5- tetra-CDPS, 2,4,4′,5-tetra-CDPS, 2,2′,3′,4,5-penta-CDPS, 2,3,3′,4,5,6-hexa-CDPS, 2,3,4,4′,5,6-hexa-CDPS, 2,2′,3,3′,4,5,6-hepta-CDPS, used in the present study were synthesized by use of palladium-catalyzed carbon–sulfur bond formation (Zhang et al., 2011). The purity (>99%) of all test PCDPSs has been identified by high pressure liquid chromatography (Agilent 1200) and 1H NMR (Bruker Avance-400 MHz) by the previous study. Stock solutions of nominal concentrations of these congeners, which ranged from 500 to 10, nmol, were prepared in dimethyl sulfoxide (DMSO; CAS number 67-68-5; >99.7% purity; Sigma-Aldrich, St. Louis, MO, USA). Cells were exposed to PCDPSs in 96-well plates. Standard solutions were in dimethylsulfoxide (DMSO). The final in-well concentration of DMSO in 96-well plates used for bioassays was 0.5%.

2.2. Cell culture and transfection with siRNA

HepG2 cells were cultured in 100-mm Petri dishes with 10 mL modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and maintained in a humid chamber at 37 °C with 5% CO_2. HepG2 cells were plated at a concentration of 10, cells per well in 96-well plates. Cells were transfected with siRNA, which target *AhR*, *CYP1A1*, using the Entranster^{TM}-R400 transfection reagent (Engreen Biosystem Co. Ltd, China) for 24 h prior to the experiment according to the manufacturers' instructions with several modifications. siRNA was purchased from GenePharma (ShangHai, China). Vehicle controls included positive siRNA and negative siRNA containing a scrambled sequence that did not lead to the specific degradation of any known cellular mRNA. The final concentrations of siRNA and Entranster TM-R400, which were contained in the medium, were 40 nM and 0.25%, respectively. Three siRNA were designed and tested for each gene, and the best one was chosen for use in the formal experiments to ensure high block efficiency.

2.3. Reactive oxygen species (ROS)

Generation of ROS was evaluated by DCFH-DA as described previously (Kopf and Walker, 2010). Briefly, DCFH-DA was prepared in DMSO as stock solution (20 mM) before the experiment. After exposure, ROS were measured at a series of time points. Cells were washed and resuspended by PBS first and were incubated with DCFH-DA (20 μM) in darkness for an additional 30 min at 37 °C with 5% CO_2. Next, the cells were plated in black-sided, clear-bottomed, 96-well plates, and the relative fluorescence was measured at the excitation wavelength of 485 nm and emission wavelength of 530 nm using a GENios plate reader (Tecan, Männedorf, Switzerland).

2.4. RNA isolation and quantitative real time-polymerase chain reaction (qPCR)

Effects of PCDPSs on gene expression, which included genes downstream in AhR-mediated pathways as well as those involved in repair of DNA, were determined by qPCR as described previously (Peng et al., 2016). Briefly, after exposure, total RNA was extracted from HepG2 cells using a Qiagen 96 RNeasy® mini kit according to the manufacturers' instructions. A FERMENTAS Kit was then used to convert total RNA into cDNA. RT-PCR was performed using the SYBR® Green PCR kit with the Applied Biosystems Stepone Plus Real-time PCR System. Conditions of the RT-PCR consisted of the following procedures: an initial denaturation step at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Expression of each target gene was normalized with the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Changes in genes expression were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Data analysis

Results of experiments were expressed as the mean ± standard deviation. Data were analyzed via one-way analysis of variance (ANOVA) using R 3.4.2. Raw data of qPCR was processed using the LinRegPCR, and fold changes of genes were subsequently calculated by the $2^{-\Delta\Delta Ct}$ method and were described as mean ± standard deviation (SD) (Livak and Schmittgen, 2001).

3. Results and discussion

3.1. PCDPSs increased ROS production in HepG2 cells

After exposure to PCDPSs for 24 h, ROS levels were detected by use of DCFH-DA. All concentrations induced a significant increase in ROS after 24 h, and levels of ROS increased in a dose-dependent manner (Fig. 1). Production of ROS varied as a function of number of Cl atoms. Less chlorinated PCDPSs has the tendency to produce more ROS in cells. For example, exposure to the highest concentration of 2,3′,4,5-tetra-CDPS, low-chlorinated PCDPSs, could cause ROS to be nearly doubled compared with the control after 24 h, whereas 2,2′,3,3′,4,5,6-hepta-CDPS could only increase ROS by a lesser percentage. This mechanism might be similar to that of PCBs, which are structurally similar to PCDPSs to a certain extent. Less-chlorinated PCBs and hydroxylated PCBs are more likely to be oxidized to quinones, which are capable of redox cycling, and producing superoxide. Superoxide can be readily converted to H_2O_2 and subsequently to highly reactive producing hydroxide radical (Dreiem et al., 2009; Spencer et al., 2009).

There is supporting evidence for the ability of PCDPSs to induce oxidative stress *in vivo*. Exposure to PCDPSs resulted in hepatic oxidative stress in fish and mice and modulated activities of superoxide dismutase (SOD) and catalase (CAT) (Li et al., 2012; Zhang et al., 2012). Furthermore, in these studies, it was found that with more Cl atoms substituted on the two benzene rings, PCDPSs exhibited greater ability to inhibit SOD and CAT, which resulted in greater production of ROS.

3.2. Effects of PCDPSs on AhR-mediated pathway

CYP1A1 is an inducible, oxidative enzyme under control of the AhR. Effects on expression of mRNA of *AhR* and *CYP1A1* were examined after exposure individually to 500, 1000, 2500, 5000 or 10, nM of each PCDPSs. Expression of CYP1A1 enzyme was significantly altered in a dose-dependent manner, except for 2,3′,4,5-tetra-CDPS. The data for CYP1A1 mRNA induction by 2,3′,4,5-tetra-CDPS can be found in Fig. 2A. It can be seen that more chlorinated PCDPS congeners tended to have greater potency to up-regulate expression of *CYP1A1* mRNA. This result is consistent with results of previous studies where the most potent congener, 2,2′,3,3′,4,5,6-hepta-CDPS, caused a 326-fold change in *CYP1A1*, whereas 2,3′,4,5-tetra-CDPS caused minor changes in expression of *CYP1A1* mRNA. This observation is consistent with results of previous *in vitro* studies where the high chlorinated congener, 2,2′,3,3’,4,5,6-hepta-CDPS, caused a 234-fold change in *Cyp1a1* mRNA while 2,4,4′,5-tetra-CDPS just caused a 21-fold change (Zhang et al., 2014a, 2016). Varying potencies to induce CYP1A1 might be due partly to the ability of more chlorinated PCDPSs to better adapt to the hydrophobic environment inside the AhR to better activate the receptor. Expression of CYP1A1 is the main bio-marker for AhR-activation. Whereas, there was no significant change in expression of the nuclear receptor protein *AhR*. These results suggest that some PCDPSs can induce expression of CYP1A1 by activation of the AhR.

However, there was no significant quantitative relationship between expression of CYP1A1 and concentrations of ROS. For example, 2,2’,4,5- tetra-CDPS led to minor changes in expression of CYP1A1, but it still produced a large amount of ROS in cells at 24 h post-treatment (Figs. 1 and 2). Also, more-chlorinated PCDPSs produced lesser amounts of ROS, but resulted in greater expression of CYP1A1. This may be due to the involvement of other oxidases such as CYP3A4. And a previous study shows that Dioxin-like compounds can cause inhibition to the CYP1A1 activity, which can also explain that (Zhan et al., 2018). A siRNA transfection assay, which was used to block the pathway, was performed to confirm the association between ROS, expression of *CYP1A1*, and activation of AhR as described below, and confirmed this result.

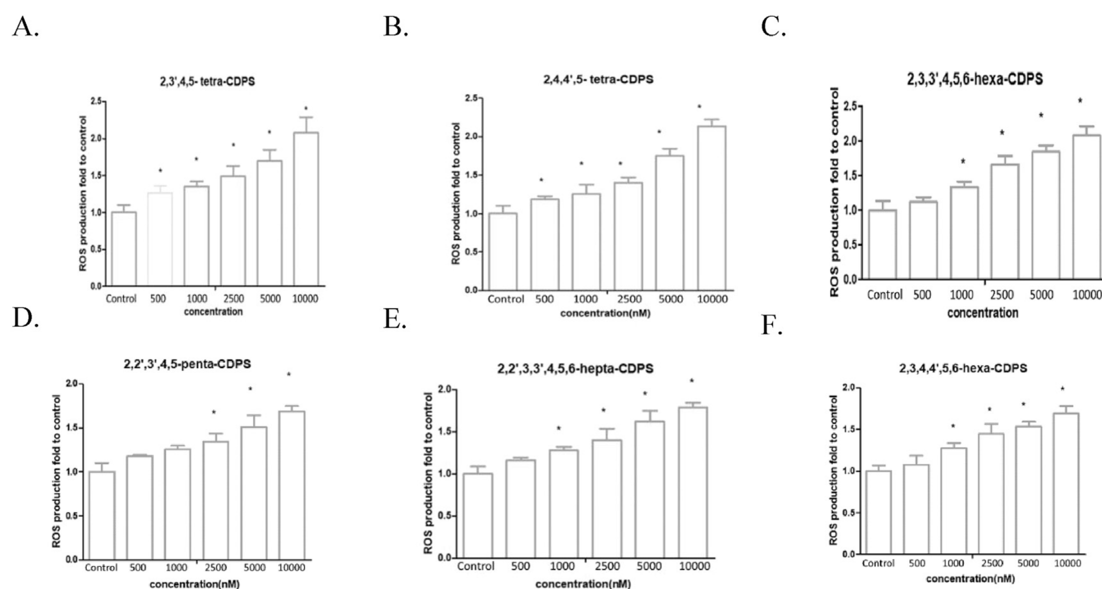

Fig. 1. HepG2 cells exposed to each of six PCDPSs for 24 h and the increase of ROS level. Results are means ± SD. of three replicates. *P < 0.05.

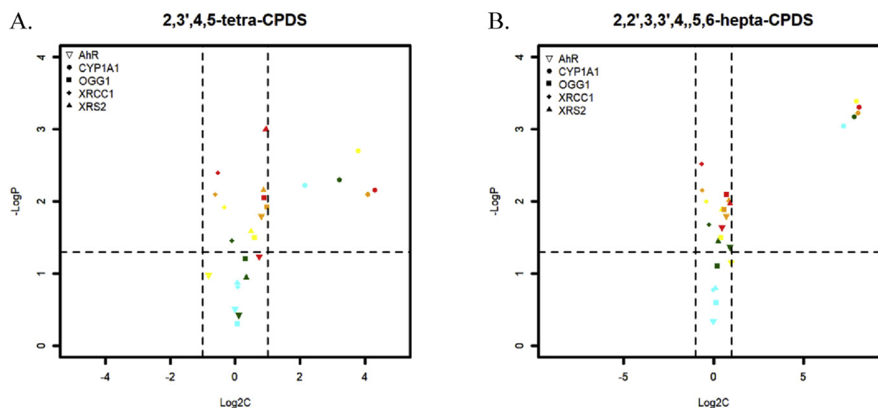

Fig. 2. Fold-changes of expressions of five genes (AhR, CYP1A1, OGG1, XRCC1 and XRS2) after exposure to PCDPSs for 24 h. Colors represent concentrations, cyan, 500; darkgreen, 1000; yellow, 2500; orange, 5000; red, 10000 nM. The fold change of RNA and significance are displayed in logarithmic form, and the bases are 2 and 10 respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Effects of PCDPSs on DNA damage

Genes involved in repair of DNA, *OGG1*, *XRS2*, *XRCC1*, which cover three DNA repair pathways, were measured in this study. These genes involved in repair of DNA have been demonstrated to play important roles in repairing DNA that had been damaged and can be used as an indicator of magnitude of genotoxicity (Lan et al., 2016). Significant increases in production of intracellular ROS probably resulted in oxidative stress and damaged DNA. Only the greatest dose of PCDPSs increased expression of mRNA for *OGG1* and *XRS2* and decrease expression of *XRCC1*, when compared with the control (Fig. 2). This result suggested the strong DNA-damaging nature of PCDPSs. Lesser concentrations of PCDPSs did not significantly up- or down-regulate expression of mRNA. These observations suggest that greater concentrations of PCDPSs can induce damage in DNA bases and DNA strands breaks that can be repaired by the base excision pathway and DNA strand break repair pathway, respectively.

To gain insight into the mechanism of DNA damage and whether DNA damage was induced by ROS caused by the AhR-CYP1A1 pathway, cells were pretreated with siRNA to knock-down expression of CYP1A1 as described below.

3.4. Suppression of CYP1A1 and AhR mRNA by siRNA

siRNA, which can block the pathway to was used in order to observe the effect of the AhR pathway on ROS and genotoxicity, and clarify relationships between CYP1A1 and ROS and DNA damage. Because of their relatively greater potencies compared with other PCDPS congeners, the most potent 2,2′,3,3’,4,5,6-hepta-CDPS was investigated selectively in the presence of siRNA. After 24 h of exposure to PCDPS, the expression of mRNA for the AhR and CYP1A1 was measured to determine the effectiveness of *CYP1A1* siRNA at reduction of expression of CYP1A1. HepG2 cells transfected with *CYP1A1* siRNA exhibited less induction of CYP1A1 compared with cells transfected with negative siRNA (Fig. 3). After treating cells with *CYP1A1* siRNA, a significant decrease in ROS (by half) was observed, which means that CYP1A1 was involved in the increase of ROS.

Similar results can also be found in HepG2 cells pretreated with *AhR* siRNA. siRNA, targeting the *AhR*, significantly reduced amounts of *AhR* mRNA in cells exposed to PCDPSs. As down-stream gene, mRNA expression of *CYP1A1* was also down-regulated with *AhR* siRNA, compared to cells transfected with negative siRNA (Fig. 3). ROS-reducing genes such as *OGG1* and *XRS2* were also suppressed

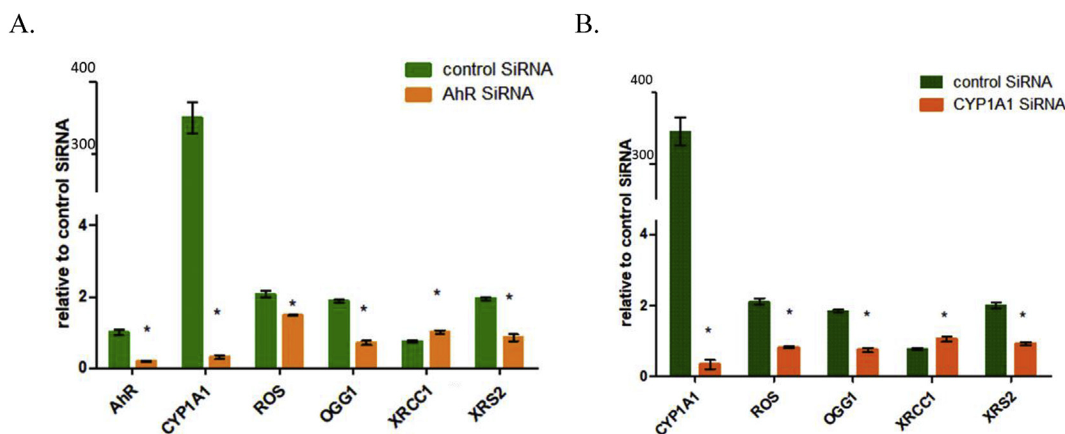

Fig. 3. HepG2 cells treated with control siRNA and siRNA target to AhR and CYP1A1. (A) The ROS level and the qPCR analysis of AhR, CYP1A1, OGG1, XRS2 and XRCC1 expression in the *AhR*-siRNA transfected cells. (B) The ROS level and the qPCR analysis of AhR, CYP1A1, ROS, OGG1, XRS2 and XRCC1 expression in the CYP1A1-siRNA transfected cells.

by *AhR* siRNA (Fig. 3). These results confirmed that AhR mediated the observed increase in ROS production.

These results further confirmed the cause-and-effect relationship between the AhR pathway and ROS production. Several previous studies also provide solid evidence that the induced levels of CYP1A1 are capable of producing ROS. By using CYP1A1-enriched microsomes, CYP1A1 has been shown previously to be responsible for production of superoxide anions and H_2O_2 (Puntarulo and Cederbaum, 1998). Microsomes from the liver of mice exposed to TCDD, produced greater amounts of superoxide than did when compared with unexposed controls or CYP1A1 null mice exposed to TCDD (Shertzer et al., 2004a, 2004b; Zangar et al., 2004).

It has long been known that ROS is the by-product of the P450 monooxygenase catalytic cycle (Zangar et al., 2004). However, in the present study, either *AhR* siRNA or *CYP1A1* siRNA did not completely prevent production of ROS. It might be that CYP1A1 was not completely knocked out and the remaining enzyme produced a large amount of ROS. Another possible explanation is that other pathways might also produce ROS, because other P450 enzymes such as CYP3A1 and CYP2B1, which were mediated by PXR and CAR, respectively can also metabolize xenobiotics (Abdelhadya et al., 2017; Tripathi et al., 2017). Further studies are needed to understand precise mechanisms of generation of ROS by PCDPSs.

3.5. Effects on OGG1, XRS2, XRCC1 by CYP1A1 and AhR siRNA

After 24 h exposure to PCDPS, cells transfected with siRNA were observed to have diminished stimulation of *OGG1* and *XRS2* mRNA accompanied by diminished *CYP1A1* mRNA expression and ROS. Expression of *XRCC1* mRNA increased with the decrease in ROS compared to cells transfected with negative siRNA.

Multiple DNA repair genes such as *OGG1*, *XRS2* and *XRCC1* have been demonstrated to participate in repair of DNA and be involved in nucleotide excision repair in mammals (Nakabeppu, 2001). These genes can repair various types of damage to DNA and are considered to functional biomarkers of exposure to DNA-damaging agents. For instance, *OGG1* is a base excision repair (BER) enzyme that recognizes and removes oxidized bases from DNA (Boiteux and Radicella, 2000). *XRS2* can respond to breaks in strands of DNA (Lan et al., 2016). Based on our data, it is likely that larger doses of PCDPSs can cause over-production of ROS and up-regulate expression of *OGG1* and *XRS2*, which means that PCDPSs can cause oxidation of bases and breaks in strands of DNA via formation of ROS. As for *XRCC1*, is used for the repair of breaks in strands of DNA and for base excision repair (Hanot-Roy et al., 2016). However, during over-production of ROS and DNA strand breaks, *XRCC1* was suppressed which means that PCDPSs can not only cause DNA strands to break, but also they might inhibit corresponding repair pathway. However, the exact mechanism of inhibition remains unknown. Considering the complicated roles of DNA repair genes in regulation of DNA damage responses, further in-depth work is needed to investigate the underlying molecular mechanisms of PCDPSs on DNA damage and repair.

4. Conclusion

PCDPSs can significantly activate the AhR pathway, and potency of activation seems to be dependent on the number of Cl atoms in PCDPSs. In HepG2 cells, exposure to most of the PCDPSs studied resulted in significant increases in ROS via the AhR pathway. However, lesser-chlorinated PCDPSs have more potency to produce ROS, which seems to be contrary to the results of the AhR-pathway activation. Furthermore, this effect can lead to oxidation of bases and breaks in strands of DNA. Results of this study strongly support the hypothesis that damage to DNA was caused through up-regulation of genes under control of the AhR. This report describes, for the first time, the mechanism of genotoxicity caused by exposure to PCDPS, and its relationship with formation of ROS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.01.169.

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Supporting Information

Polychlorinated diphenyl sulfides induce ROS via the AhR-CYP1A1 pathway and cause genotoxicity

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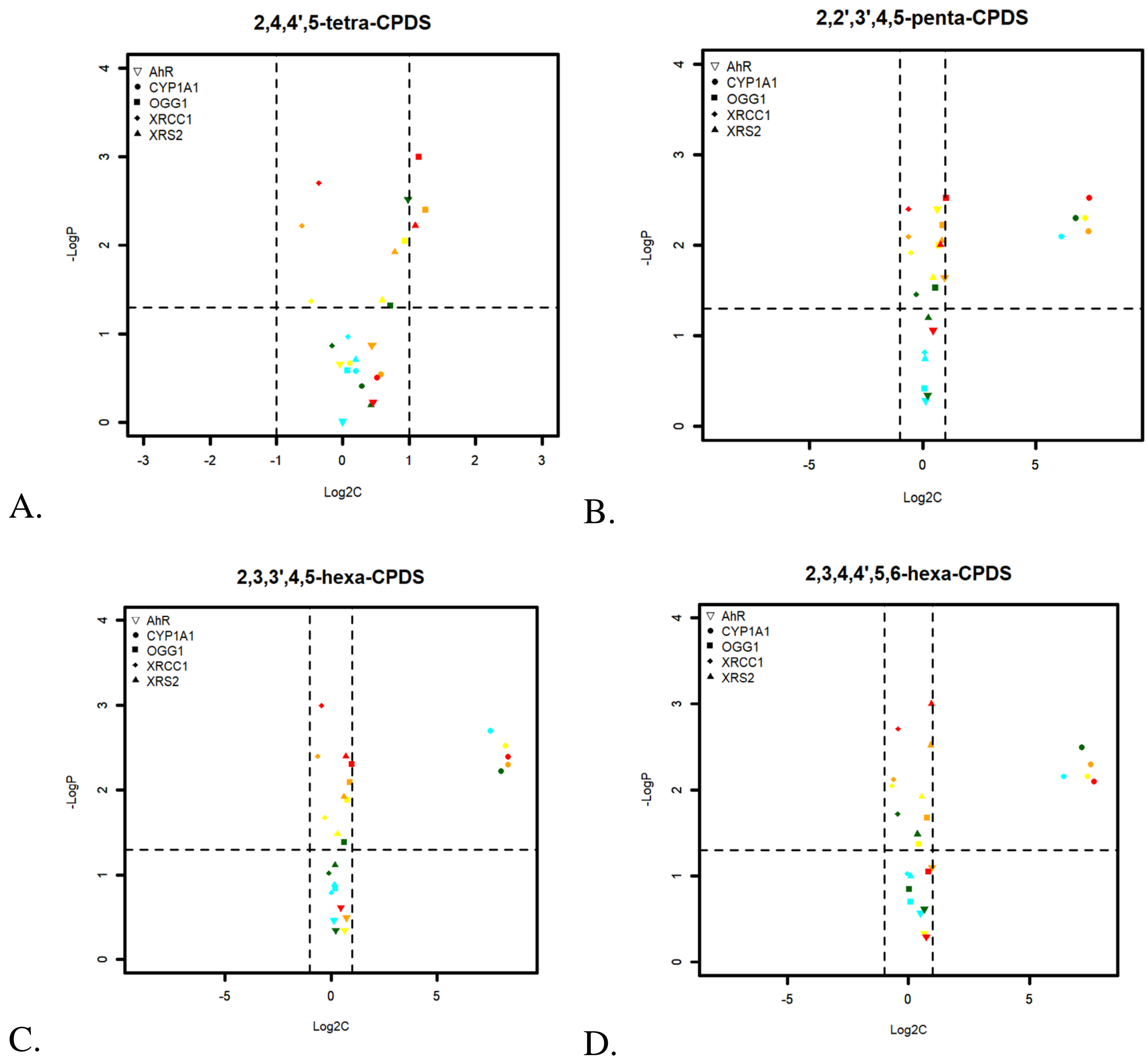

Fig. S1. Fold-changes of expressions of five genes associated with AhR, CYP1A1, OGG1, XRCC1 and XRS2 after exposure to PCDPSs for 24 h. Colors represent concentrations: cyan, 500 nM; darkgreen, 1000 nM; yellow, 2500 nM; orange, 5000 nM; red, 10000 nM.

Table S1. Sequences of AhR related genes used as primers for qRT-PCR.

Genes	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Gene ID
ahr	TCCCCATACCCCACTACTTC	TTCTGGCTGGCACTGATACA	NM_001621
cyp1a1	CCTATTCTTCGCTACCTACCC	TGGACATTGGCGTTCTCAT	NM_001319217
ogg1	TGGACCTGGTTCTGCCTTCT	TGGCTCTTGTCTCCTCGGTA	NM_016829
xrs2	CGCACTAAAGCCGACTGAA	GACTGTAGCGACTTATCCAACG	AF069291
xrcc1	GATGGGGAACAGTCAGAAGGAC	AATTGGCAGGTCAGCCTCTG	NM_006297